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RESEARCH****Research Report****Chronic activation of the 5-HT₂ receptor reduces 5-HT neurite density as studied in organotypic slice cultures**

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ABSTRACT

The serotonin system densely innervates the brain and is implicated in psychopathological processes. Here we studied the effect of serotonin and serotonin pharmacological compounds on the outgrowth of serotonergic projections using organotypic slice co-cultures of hippocampus and dorsal raphe nuclei. Immunocytochemical analysis showed that several serotonergic neurites had grown into the target slice within 7 days in culture, after which the neurite density stabilized. These projections expressed the serotonin-synthesizing enzyme Tryptophan hydroxylase and the serotonin transporter and contained several serotonin-positive varicosities that also accumulated presynaptic markers. Chronic application of a 5-HT₂ agonist reduced the serotonergic neurite density, without effects on survival of serotonergic neurons. In contrast, application of a 5-HT_{1A} agonist or the serotonin transporter inhibitor fluoxetine did not affect serotonergic neurite density. We conclude that serotonergic connectivity was reproduced in vitro and that the serotonin neurite density is inhibited by chronic activation of the 5-HT₂ receptor.

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1. Introduction

The serotonin (5-hydroxytryptamine (5-HT)) system has its cell bodies clustered in the midbrain raphe nuclei and sends abundant projections to virtually every brain area (Hornung, 2003; Rubenstein, 1998). The 5-HT system influences a wide variety of physiological processes, such as sleep–wake

rhythm, feeding, sexual behavior and nociception (Jacobs and Azmitia, 1992). Moreover, the 5-HT system is implicated in several psychopathological processes, such as anxiety, aggression, obsessive–compulsive disorder and depression (Castren, 2005).

During brain development, the 5-HT neurons are one of the first groups of cells expressing a specific neurotransmitter

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Abbreviations: 5-HT, serotonin; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetraline; DIV, days in vitro; dGBSS, dissection Gey's balanced salt solution; DOI hydrochloride, (+)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; DRN, dorsal raphe nucleus; MAOA, monoamine oxidase A; SERT, 5-HT reuptake transporter; Tph, Tryptophan hydroxylase

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(Gaspar et al., 2003; Lauder, 1990). Accumulating evidence shows that alterations in brain 5-HT levels during development could result in alterations in brain development and behavioral alterations. 5-HT reuptake transporter (SERT) knockout mice have an increase in extraneuronal 5-HT (Bengel et al., 1998; Mathews et al., 2004). They exhibit behavioral alterations, reduced number of 5-HT neurons in the dorsal raphe nuclei (DRN) and reduced firing of the 5-HT neurons in the DRN (Lira et al., 2003). These behavioral alterations observed in SERT knockout mice can be mimicked by application of the SERT inhibitor fluoxetine during development between postnatal days 4 and 21 (Ansorge et al., 2004).

In contrast to reduction in brain 5-HT content after disruption of the SERT gene, there is a significant increase in brain 5-HT levels after inactivation of the monoamine oxidase A (MAOA) gene (Cases et al., 1996). The chronic elevation of 5-HT levels during brain development results in lack of barrels in the barrel cortex (Cases et al., 1996). These data suggest that either reductions in brain 5-HT levels or excess 5-HT levels during a critical period in the development of the brain can lead to abnormalities in brain development and behavioral alterations. In vitro experiments have shown that 5-HT can inhibit the outgrowth of neurites or even cause growth cone collapse on 5-HT neurons (Haydon et al., 1984; Koert et al., 2001). Whether alterations in brain 5-HT levels during development of the brain result in alterations in 5-HT outgrowth, branching or connectivity or whether the behavioral alterations observed are a result of alterations in the 5-HT outgrowth and connectivity is currently unknown. Organotypic slice cultures of the DRN and a target slice could be a valuable reduced model system to study 5-HT outgrowth in vitro.

Therefore, the aim of this study was to investigate whether organotypic slice co-cultures of DRN and hippocampus, a method that has previously been characterized, can be used to study 5-HT outgrowth and the effect of pharmacological manipulations in vitro (Guthrie et al., 2005; Papp et al., 1995). We show that within 7 days of culturing several 5-HT neurites have grown into the hippocampal slice. Using these slice co-cultures, we studied whether chronic application of 5-HT pharmacological compounds affects the outgrowth of 5-HT neurites from the DRN. To this end, we used a 5-HT_{1A} receptor agonist, a 5-HT₂ receptor agonist and the SERT inhibitor fluoxetine since it has been shown before, also on non-5-HT cells, that (chronic) activation of a 5-HT_{1A} or 5-HT₂ receptor or blockade of the SERT can affect (5-HT) outgrowth or synaptogenesis (Fricker et al., 2005; Kondoh et al., 2004; Wilson et al., 1998; Zhou et al., 2006). We show here that chronic application of a 5-HT₂ agonist results in a reduction in 5-HT neurite density. We conclude that these organotypic slice co-cultures can be used to study 5-HT outgrowth and the effect of 5-HT pharmacological compounds on this outgrowth in vitro.

2. Results

2.1. Development and outgrowth of 5-HT neurons in organotypic slice co-cultures

To study the outgrowth of 5-HT neurites ex vivo, we used organotypic slice co-cultures of the DRN and a hippocampal

slice. We positioned the slices close to each other to allow 5-HT neurites to grow into the hippocampal slice. Within 7 days of culturing the slices flattened out and several non-neuronal cells migrated away from the slices (Fig. 1). We performed immunocytochemistry for 5-HT to investigate whether 5-HT neurons survived in the slices. This showed that in several DRN slices 5-HT neurons were present, which resembled the topology of the 5-HT neurons in the DRN in vivo (Fig. 2A). However, there were also DRN slices that contained only few 5-HT neurons, possibly resulting from a variation in the dissection of the DRN slice. A Z-stack of 5-HT cell somata showed that these cells have several primary neurites that contain numerous varicosities (Fig. 2B). In the growth cones of 5-HT neurites, 5-HT was also detected (Fig. 2C). Next, we focused on the expression of Tph and SERT, two markers for the 5-HT system. In 5-HT-immunopositive cell bodies and in the 5-HT neurites and varicosities, Tph was also expressed (Figs. 2D and E). We found that all the 5-HT neurites were positive for SERT labeling, with the highest level of SERT staining on the soma and in the 5-HT varicosities (Figs. 2F and G). Moreover, SERT was also expressed in a punctuate pattern in the 5-HT growth cones (Fig. 2H). In vivo, 5-HT is released from varicosities in 5-HT axons and dendrites and 5-HT release is predominantly paracrine (Bunin and Wightman, 1999). In the slices, the 5-HT neurites contained several round and fusiform shaped varicosities (Figs. 3A and B). Immunocytochemistry revealed that in these varicosities, the calcium sensor synaptotagmin and the active zone marker Bassoon are present, suggesting that the varicosities are presynaptic sites where calcium-dependent 5-HT release occurs (Figs. 3C and D; Perin et al., 1991; tom Dieck et al., 1998). This showed

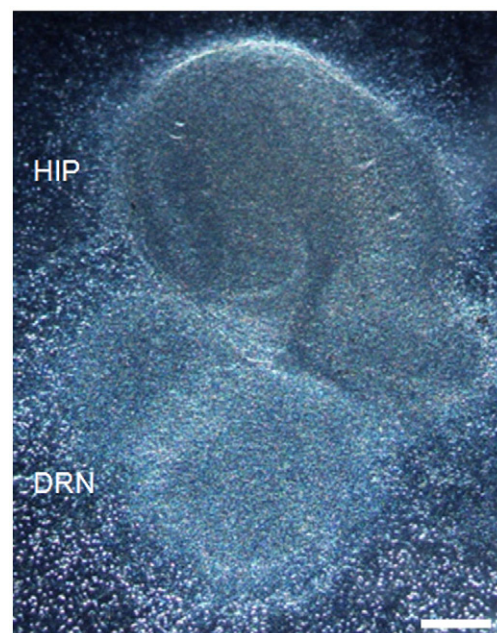


Fig. 1 – Overview of the culturing method. The DRN and hippocampal slice were positioned close to each other to allow 5-HT neurites to grow into the hippocampal slice. After culturing for 7 days, the slices flatten and several cells (e.g. macrophages) migrate away from the slices. Scale bar: 500 μ m.

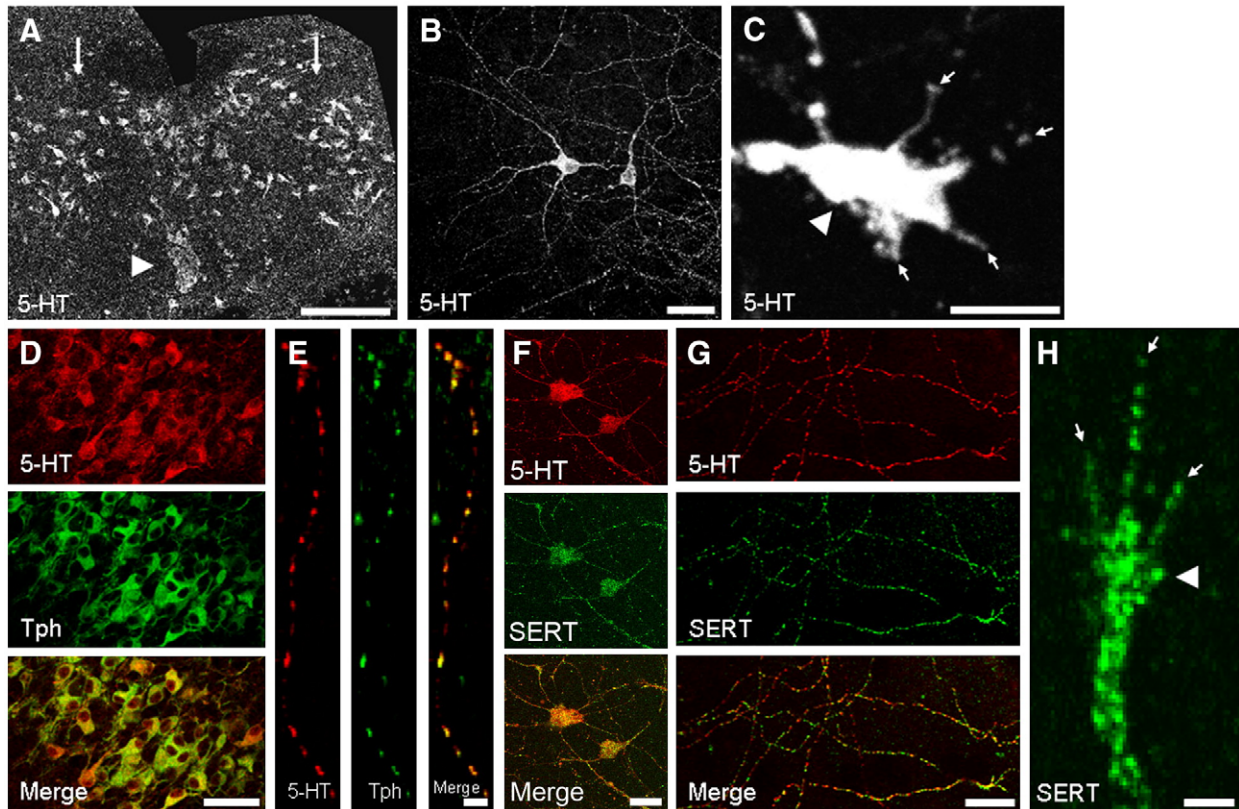


Fig. 2 – 5-HT neurons survive in the slice and grow out several 5-HT neurites. (A) A DRN slice cultured for 7 days which is stained for 5-HT shows that several 5-HT cells are present in the slice. Note the presence of the lateral wings of the DRN (arrows) and the group of 5-HT neurons in the ventral part of the DRN (arrowhead). **(B)** A Z-stack of two cells shows that the cells project several neurites that are 5-HT positive. **(C)** The growth cones also contain 5-HT, both in the core (arrowhead) and in the filopodia (arrows). **(D, E)** Staining for the rate limiting enzyme Tph shows that Tph is present both in the cell bodies and in the varicosities in the neurites. **(F, G)** The other marker for the 5-HT system, SERT, is present in 5-HT cell bodies and 5-HT neurites. **(G)** Moreover, there is also a punctuate SERT staining in 5-HT growth cones, both in the core (arrowhead) and in the filopodia (arrows). Scale bars: 200 μm in A, 50 μm in B and D, 20 μm in F and G, 5 μm in C and E and 2 μm in H.

that in organotypic slice co-cultures of DRN and hippocampus, 5-HT neurons survive and grow out neurites that contain 5-HT varicosities with immunoreactivity for all tested components of the 5-HT release machinery.

2.2. Dense ingrowth of 5-HT neurites into hippocampal slices

To study the outgrowth, we fixed slice cultures at different time points and quantified the 5-HT outgrowth. To this end, the images were converted to binary images and the 5-HT neurite density in the slices was quantified (Figs. 4A–C). We quantified the 5-HT neurite density as the area in the slice occupied by 5-HT neurites compared to the total area of the slice. Immunocytochemistry for 5-HT on hippocampal slices cultured without the DRN for 7 days revealed that there were no 5-HT neurites present (Fig. 4E). Thus, 5-HT neurites observed in the hippocampal slice in co-cultures are the result of re-growth of 5-HT neurites from the DRN slice. In co-cultures fixed at DIV4, we observed that the DRN slice already contained several 5-HT neurites, and the first 5-HT neurites started to grow into the hippocampal slice (Figs. 4F and I). At DIV7, also the hippocampal slice contained several 5-HT

neurites (Figs. 4G and J). Quantification of the 5-HT neurite density in the slices at DIV14 and DIV21 revealed that this was not significantly different from DIV7 (Figs. 4D and H; DIV7 DRN $8.42\% \pm 0.8\%$, HIP $6.31\% \pm 0.84\%$, $n=16$; DIV14 DRN $9.49\% \pm 1.42\%$, HIP $5.12\% \pm 0.9\%$, $n=12$; DIV21 DRN $9.26\% \pm 1.16\%$, HIP $6.61\% \pm 1.43\%$, $n=4$). We investigated whether there is regional variation in the 5-HT neurite distribution in the hippocampus. At DIV4, the first 5-HT neurites that start growing into the hippocampal slice do not appear to display a preference for a certain region (Fig. 4K). At DIV7, 5-HT neurites have grown in a uniform distribution into the hippocampus, and there are no regions that contain a higher density of 5-HT neurites than other regions (Fig. 4L). Thus, the initial outgrowth of the 5-HT neurites occurs in the first 7 days, after which the network stabilizes, and the homogenous distribution suggests that 5-HT neurites do not have any preferential target region within the hippocampal slice in vitro.

2.3. The effect of 5-HT pharmacological compounds on the outgrowth of 5-HT projections

Finally, we studied whether this culture model system can be used to study the effect of chronic application of 5-HT

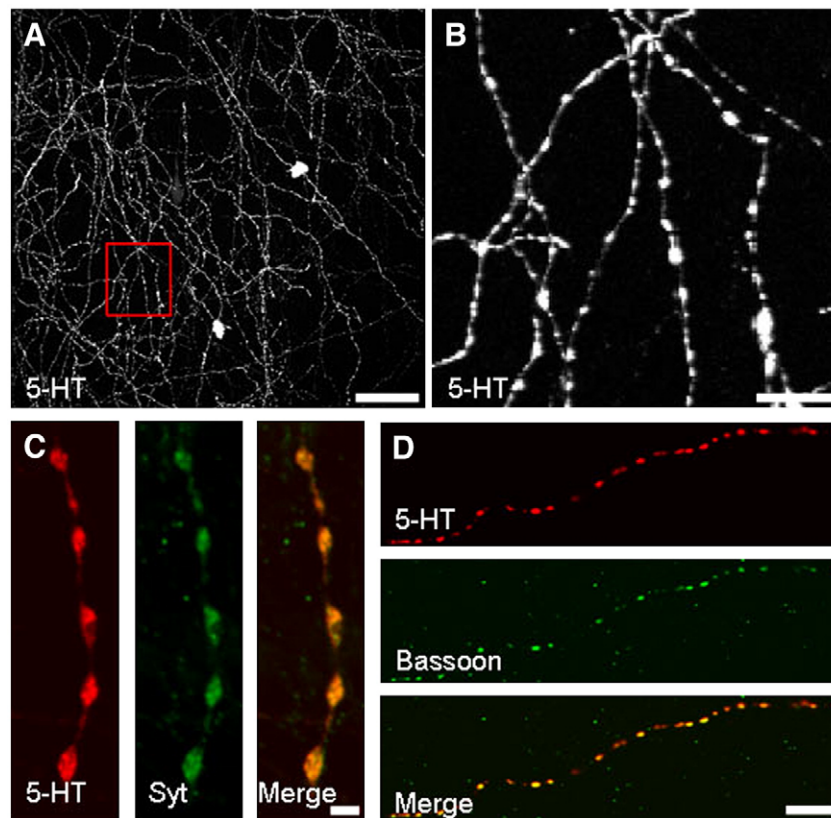


Fig. 3 – 5-HT neurites contain several varicosities which are positive for presynaptic markers. (A) A Z-stack in the hippocampal slice shows that several thin, highly branching 5-HT neurites have grown into the slice. **(B)** A blow-up of the red box in A shows that these neurites contain several varicosities. **(C, D)** These varicosities are positive for the protein involved in calcium-dependent secretion, synaptotagmin (Syt) and for the active zone marker Bassoon. Scale bars: 50 μm in A, 10 μm in B and D and 2 μm in C.

pharmacological compounds on the outgrowth of 5-HT neurites. There are several 5-HT receptors that are expressed in both the DRN and the hippocampus. These include the 5-HT_{1A} receptor, the 5-HT₂ receptor and the 5-HT₇ receptor (Bickmeyer et al., 2002; Clemett et al., 2000; Garcia-Alcocer et al., 2006; Gustafson et al., 1996; Xu and Pandey, 2000). Since it has been shown before, also on non-5-HT cells, that (chronic) activation of a 5-HT_{1A} or 5-HT₂ receptor or blockade of the SERT can affect (5-HT) outgrowth or synaptogenesis, we decided to use a 5-HT_{1A} receptor agonist (8-OH-DPAT), a 5-HT₂ receptor agonist (DOI hydrochloride) and the SERT inhibitor fluoxetine (Fricker et al., 2005; Kondoh et al., 2004; Wilson et al., 1998; Zhou et al., 2006). We chronically applied the pharmacological compounds in 10 μM concentrations during the first 7 days of outgrowth.

First of all, we studied the effect of chronic blockade of the SERT by fluoxetine. Fluoxetine did not affect 5-HT neurite density (fluoxetine: DRN, 8.44 ± 0.72 ; HIP, 6.12 ± 1.12 ; $n=10$) compared to control condition (Figs. 5A, B, E, F). Subsequently, we tested the effect of chronically activating the 5-HT_{1A} and 5-HT₂ receptor, two receptors that are present on 5-HT neurons. Moreover, it has been shown previously that activation of these receptors can affect outgrowth of Purkinje cells (Kondoh et al., 2004). First we investigated the effect of

the 5-HT_{1A} receptor agonist 8-OH-DPAT. Activation of the autoreceptor by 5-HT or an agonist results in a reduction in 5-HT release (Quick, 2003). However, 8-OH-DPAT did not affect 5-HT neurite density (DRN, $10.67 \pm 0.79\%$; HIP, $7.21 \pm 0.9\%$; $n=9$; Figs. 5C, E, F).

Next, we investigated the effect of chronic 5-HT₂ receptor activation. Activation of the 5-HT₂ receptor results in an increased protein kinase C activity and release of calcium from internal stores. We chronically activated the 5-HT₂ receptor by applying the 5-HT₂ receptor agonist DOI hydrochloride. Chronic application of the 5-HT₂ receptor agonist DOI hydrochloride resulted in a significantly decreased 5-HT neurite density (Figs. 5D–F; DRN, $2.94 \pm 1.01\%$; HIP, $2.29 \pm 1.05\%$; $n=5$). To exclude the possibility that pharmacological treatment affected the survival of 5-HT neurons, we counted the number of 5-HT cells per group. This showed that treatment with fluoxetine, 8-OH-DPAT or DOI hydrochloride did not affect the number of 5-HT neurons in the slices (data not shown). Hence, changes in viability of 5-HT neurons cannot explain the effect of DOI hydrochloride. We conclude that these organotypic slice co-cultures can be used to study the 5-HT outgrowth and ingrowth into a target area in vitro and that chronic activation of the 5-HT₂ receptor results in a decreased 5-HT neurite density.

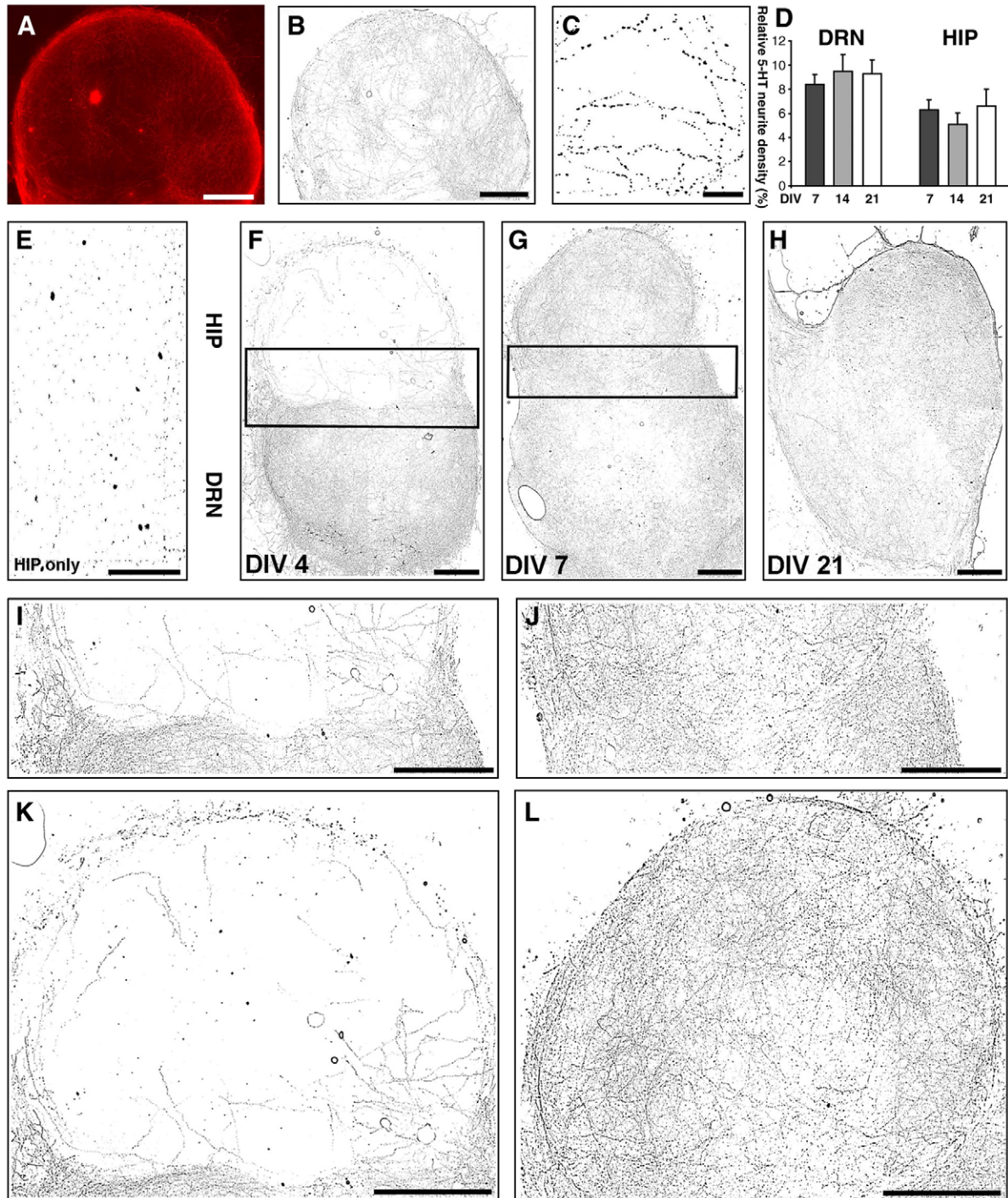


Fig. 4 – Dense ingrowth of 5-HT neurites into hippocampal slices within 7 days of culturing. An original image of a hippocampal slice co-cultured with a DRN slice shows that there is abundant ingrowth of 5-HT neurites, but there is also a lot of red background staining. (B) When this image is converted to a binary image using the steps described in Experimental procedures, the 5-HT neurites are positively labeled (black), whereas the background is removed (white). (C) A blow-up of part of the binary image shows that all 5-HT neurites are detected, and the red background is removed. (D) Quantification of the 5-HT neurite density (Y-axis) in the DRN and hippocampal slices at DIV 7, 14 and 21. (E) A hippocampal slice cultured without the DRN contains only background staining and no 5-HT neurites. (F, I) Maximal 5-HT ingrowth of the slices is reached in the first 7 days of culturing. At DIV4, the DRN slice already contains several 5-HT neurites, and the first 5-HT neurites start to grow into the hippocampal slice. (G, J) At DIV7, both the DRN and hippocampal slice contain abundant 5-HT neurites. (H) At DIV21, the 5-HT neurite density in the slices has not changed. (K, L) No regional variation in 5-HT neurite density in the hippocampal slice at DIV4 (K) and DIV7 (L). Panels I and J are a blow-up of the black box in panels F and G, respectively. Panels K and L are blow-ups of the hippocampal slices in panels F and G, respectively. Scale bars: 500 μ m in A, B, E, F, G, H, I, J, K and L, 20 μ m in C.

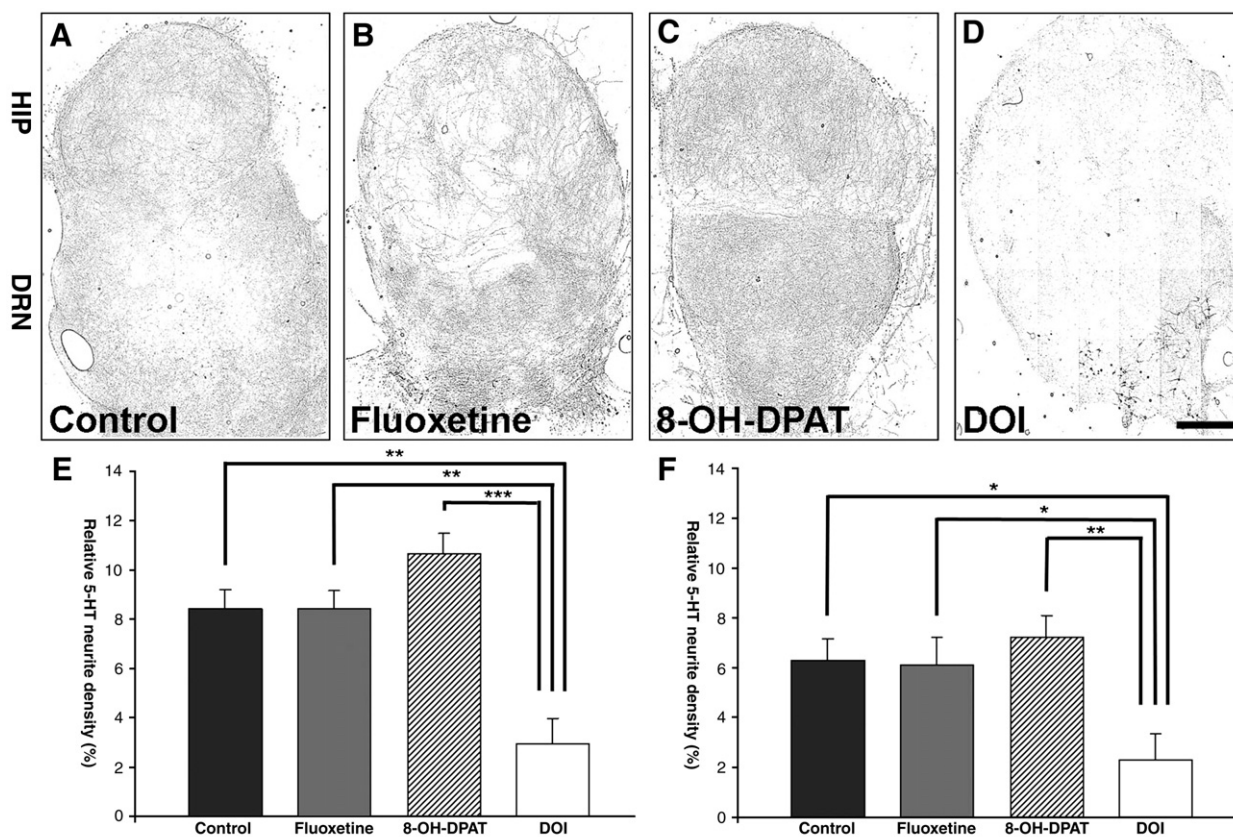


Fig. 5 – Chronic application of a 5-HT₂ agonist reduces 5-HT neurite density in the slices. (A) In control condition, after 7 days of culturing several 5-HT neurites have grown into the slices. **(B)** Chronic blockade of the SERT with the SERT inhibitor fluoxetine did not affect 5-HT neurite density in the slices. **(C)** Chronic activation of the 5-HT_{1A} receptor with 8-OH-DPAT also did not affect 5-HT neurite density in the slices. **(D)** However, chronic activation of the 5-HT₂ receptor with DOI significantly reduced 5-HT neurite density both in the DRN and hippocampal slice. **(E, F)** Quantification of 5-HT neurite density in the DRN slice and hippocampal slice, respectively. Scale bar: 500 μ m. * p <0.05, ** p <0.01, *** p <0.001.

3. Discussion

3.1. An organotypic co-culture model system to study 5-HT outgrowth ex vivo

We used organotypic slice co-cultures to study the outgrowth of the 5-HT system ex vivo. Neurite projections of 5-HT neurons grew into the slices, and at 7 days a dense network had formed with many serotonergic boutons that had accumulated presynaptic markers. The regional differences in 5-HT innervation observed in the hippocampus in vivo were not reproduced ex vivo, and it has been observed that fewer synapses form in slices than in vivo (see discussion in Papp et al., 1995). However, also in vivo the majority of the 5-HT release sites (70–80%; Oleskevich and Descarries, 1990) are not classical synapses, i.e., with a juxtaposed postsynaptic specialization. Hence, the in vivo connectivity of the 5-HT system appears to depend largely on non-synaptic release. Therefore, despite some discrepancies compared to the in vivo situation, the slice co-culture system is an excellent, reduced model to unravel the basic principles that orchestrate 5-HT neurite outgrowth and connectivity. Such a reduced system is a valuable supplementation of in vivo studies, which are often compli-

cated by the exceptionally complex and dynamic connectivity of the 5-HT system, with for instance large fluctuations in innervation over the course of a few days (transient hyperinnervation, see Fujimiya et al., 1986; D'Amato et al., 1987).

3.2. Chronic activation of the 5-HT₂ receptor decreased 5-HT neurite density

We found that chronic application of a 5-HT₂ agonist decreased the neurite density of 5-HT neurites. Since DOI hydrochloride treatment did not affect 5-HT neuron survival, 5-HT-mediated cytotoxicity cannot explain the decrease in 5-HT neurite density. Hence, the decreased neurite density appears to be the result of a specific effect of the activation of the 5-HT₂ receptor on the neurites (decreased outgrowth/increased pruning). In contrast, chronic activation of the 5-HT_{1A} receptor did not mimic the effects of 5-HT, although the 5-HT_{1A} receptor has often been implicated in shaping 5-HT connectivity (Gross et al., 2000, 2002). Activation of somato-dendritic 5-HT_{1A} receptors results in hyperpolarization and a decrease in 5-HT release (Quick, 2003). However, chronic activation results in rapid desensitization (Assie et al., 2006), which may explain why we do not observe an effect of chronic 5-HT_{1A} receptor activation. On the other hand, 5-HT₂ receptor

activation increases intracellular calcium and activates protein kinase C (Tamir et al., 1992). This suggests that (chronic) activation of the 5-HT₂ receptor affects outgrowth since elevation of calcium levels in growth cones reduces outgrowth (Gomez and Spitzer, 1999). Since DOI hydrochloride has comparable affinity for the 5-HT_{2A} and 5-HT_{2C} receptor subtype, based on these data we cannot conclude which of the two subtypes is involved in the effect of DOI hydrochloride.

However, although the 5-HT_{2A} receptor is widely expressed throughout the brain, the receptor seems not to be expressed in the DRN (Xu and Pandey, 2000). In contrast, the 5-HT_{2C} receptor is present in the DRN (Clemett et al., 2000; Serrats et al., 2005). Moreover, it has been shown previously that activation of the 5-HT_{2C} receptor in the DRN results in a reduction in 5-HT neuron firing (Boothman et al., 2006). Therefore, based on the anatomical distribution, we speculate that the effect of DOI hydrochloride is predominantly mediated via the 5-HT_{2C} receptor. Fluoxetine did not decrease 5-HT neurite density. It could be expected that fluoxetine application mimics a 5-HT_{1A} receptor agonist, i.e., SERT blockade results in an increase in extracellular 5-HT that could activate 5-HT_{1A} autoreceptors. However, possibly upon application of fluoxetine extracellular 5-HT is rapidly diluted in the culture medium *in vitro*, and therefore extracellular 5-HT will not reach high enough concentration levels to mimic the effect of a 5-HT_{1A} receptor agonist. Also chronic fluoxetine treatment *in vivo* failed to produce effects similar to chronic 5-HT application (Zhou et al., 2006). In fact, chronic fluoxetine enhanced neurite density in this case (Zhou et al., 2006).

3.3. Conclusions

We have shown here that in DRN, hippocampus organotypic slice co-cultures 5-HT projections grow into the target slice. After 4 days of culturing in the DRN slice already several 5-HT projections were present, and the first 5-HT projections started to grow into the target slice. Within 7 days of culturing also the target slice contained several 5-HT neurites. These 5-HT neurites contained several varicosities, which are presumably sites of 5-HT release since presynaptic markers were present in these varicosities. Finally, we have shown that this culture model system can be used to study the effect of pharmacological manipulations on the outgrowth of 5-HT neurites and that application of a 5-HT₂ agonist results in a reduction in 5-HT neurite density in the slices.

4. Experimental procedures

4.1. Laboratory animals

Wild-type C57BL/6 mouse fetuses were obtained from caesarean section at embryonic day 18 or postnatal day 1. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

4.2. Pharmacological compounds and antibodies

Mouse monoclonal anti-tryptophan hydroxylase (Tph) antibody, which detects both Tph1 and Tph2, was obtained

from Sigma-Aldrich and used in a 1:1000 dilution. Rabbit anti-5-HT polyclonal and mouse anti-SERT monoclonal antibodies were obtained from Immunostar/Diasorin and used in a 1:1000 dilution. Bassoon and synaptotagmin monoclonal antibodies were used in a 1:500 and 1:1000 dilutions, respectively, and obtained from Stressgen. As secondary antibodies, Alexa593-conjugated goat anti-rabbit and Alexa488-conjugated goat anti-mouse were used in a 1:1000 dilution (Molecular probes). 8-Hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT) and (+)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI hydrochloride) were obtained from Sigma-Aldrich. Fluoxetine was obtained from Tocris.

4.3. Organotypic slice co-cultures

Organotypic slice cultures were made as follows. After decapitation of the fetuses, the heads were immediately transferred to ice-cold dissection Gey's balanced salt solution (dGBSS, Invitrogen, supplemented with 0.65 g/l glucose and 200 μ M kynurenic acid). For isolation of a DRN slice, the brains were dissected in dGBSS and the midbrain cut into 400 μ m thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall, UK). Individual slices were separated in dGBSS and the hindbrain slice containing the rostral DRN was identified by visual inspection. The DRN was dissected out using a dissection knife. The entire hippocampus was dissected out of the brain and individual slices (400 μ m) were sagittally cut perpendicular to the hippocampus' longitudinal axis. The hippocampal and DRN slices were allowed to recuperate in dGBSS at 4 °C for 60 min. A hippocampal and DRN slice were cultured in close proximity on a poly-D-lysine coated 12×24 glass coverslip (O. Kindler GmbH & Co. Mikroskopische Gläser, Freiburg, Germany) in a plasma clot (chicken plasma; Cocalico Biologicals Inc. Reamstown, U.S.), which was coagulated with thrombin (Merck, Darmstadt, Germany). Culturing medium consisted of 50% BME Hanks, 25% Hanks balanced salt solution and 25% horse serum supplemented with 1.3 g/l glucose and 200 μ M glutamine. For culturing, 700 μ l of culturing medium was added to the slices in a Nunc flat-bottomed tube. The slices were incubated in a roller drum at 36 °C. After the first week, medium was changed twice a week.

4.4. Pharmacological treatments

All pharmacological compounds were dissolved in ddH₂O, filter sterilized and aliquots stored at –80 °C. After 1 day *in vitro* (DIV), 10 μ M of the pharmacological compound was added to the slices. Every day, a new aliquot of pharmacological compound was added to the slices. In control slices, only vehicle was added (sterile ddH₂O). At DIV7, the slices were fixed, processed and analyzed as described below.

4.5. Immunocytochemistry

Slices were fixed for 20 min in 4% paraformaldehyde dissolved in phosphate-buffered saline. After fixation, the slices were washed three times for 5 min in PBS, and nonspecific binding was blocked by incubating the slices in

PBS containing 0.1% Triton X-100 and 2% normal goat serum for 2 h. The slices were incubated overnight with the primary antibodies diluted in phosphate-buffered saline containing 0.1% Triton X-100 supplemented with 2% normal goat serum at 4 °C. After incubation in the primary antibodies, the slices were washed three times for 2 h in phosphate-buffered saline on a shaking platform. The secondary antibodies were diluted in phosphate-buffered saline supplemented with 2% normal goat serum and incubated for 1 h. After incubation in the secondary antibodies, the slices were washed again three times for 2 h in phosphate-buffered saline and mounted in Dabco-Mowiol (Sigma) on glass coverslips. All reactions were carried out at room temperature unless otherwise stated. No labeling was observed when omitting the primary antibodies.

4.6. Confocal analysis

Confocal analysis of the slices was performed on a LSM 510 microscope (Carl Zeiss b.v. Weesp, the Netherlands) and a 63× Plan-Neofluar lens (Numerical aperture 1.4, Carl Zeiss). To excite the 488 antibody, a HeNe1 laser was used and for excitation of the 593 antibody a HeNe2 laser was used. For analysis of the 5-HT neuron morphology and the 5-HT neurites, Z-stacks of 1 µm were made. Images were analyzed and further processed in Zeiss CLSM software.

4.7. Histological quantification of 5-HT neurite density

Quantification of 5-HT-immunopositive neurites and cells was performed using an MCID Elite imaging system (Imaging Research Inc., Ontario, Canada). Images of the immunostained co-cultures were digitized using an objective magnification of 20× on a Leica DM/RBE photo-microscope with a Sony (DXC-950P, 640×512 pixels) camera using epifluorescence microscopy. The 5-HT-immunopositive neurites were segregated from background using several point operators and spatial filters combined in an algorithm designed to detect local changes in relative optical density. Briefly, images underwent histogram equalization and smoothing (low-pass filter, kernel size 7×7). The unfiltered image was subtracted from the smoothed image, followed by a series of steps to optimize the processed image and make it a suitable measuring template for detecting objects the size and shape of 5-HT neurites or cell bodies. This algorithm was preferred over relative optical density thresholding since it does not involve an observer-dependent operation. Finally, a line was manually drawn around the DRN or hippocampal slice, and the number of positive pixels (i.e., pixels representing 5-HT neurites) compared to the total number of pixels was calculated. The number of 5-HT neurons was counted manually in the slices.

4.8. Data analysis

In order to analyze differences in neurite density between different pharmacological treatments, ANOVA was used with the Bonferroni test for post hoc analysis. Data shown are mean values ± standard errors of mean. Significance levels were set at <0.05.

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